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| (54) Title: ANTISENSE MODULATION OF TNFR1 EXPRESSION | | |
| (57) Abstract <p>Antisense compounds, compositions and methods are provided for modulating the expression of TNFR1. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding TNFR1. Methods of using these compounds for modulation of TNFR1 expression and for treatment of diseases associated with expression of TNFR1 are provided.</p> | | |

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-1-

ANTISENSE MODULATION OF TNFR1 EXPRESSION**FIELD OF THE INVENTION**

The present invention provides compositions and methods of modulating the expression of TNFR1. In particular, this invention relates to antisense compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding human TNFR1. Such oligonucleotides have been shown to modulate the expression of TNFR1.

BACKGROUND OF THE INVENTION

One of the principal mechanisms by which cellular regulation is effected is through the transduction of extracellular signals into intracellular signals that in turn modulate biochemical pathways. Examples of such extracellular signaling molecules include growth factors, cytokines, and chemokines. The cell surface receptors of these molecules and their associated signal transduction pathways are therefore one of the principal means by which cellular behavior is regulated. Because cellular phenotypes are largely influenced by the activity of these pathways, it is currently believed that a number of disease states and/or disorders are a result of either aberrant activation or functional mutations in the molecular components of signal transduction pathways.

For example, the polypeptide cytokine tumor necrosis factor (TNF) is normally produced during infection, injury, or invasion where it serves as a pivotal mediator of the inflammatory response. In recent years, a number of in vivo animal and human studies have demonstrated that overexpression TNF by the host in response to disease and infection is itself responsible for the pathological consequences associated with the underlying disease. For example, septic shock as a result of massive bacterial infection has been attributed to infection-induced expression of TNF. Thus, systemic exposure to TNF at

-2-

levels comparable to those following massive bacterial infection has been shown to result in a spectrum of symptoms (shock, tissue injury, capillary leakage, hypoxia, pulmonary edema, multiple organ failure, and high mortality rate) that is virtually indistinguishable from septic shock syndrome [Tracey, 1994 #7415]. Further evidence has been provided in animal models of septic shock, in which it has been demonstrated that systemic exposure to anti-TNF neutralizing antibodies block bacterial-induced sepsis [Tracey, 1994 #7415]. In addition to these acute effects, chronic exposure to low-dose TNF, results in a syndrome of cachexia marked by anorexia, weight loss, dehydration, and depletion of whole-body protein and lipid. Chronic production of TNF has been implicated in a number of diseases including AIDS and cancer [Tracey, 1994 #7415]. To date, two distinct TNF cells surface receptors, known as TNFR1 and TNFR2, have been described. Molecular analysis of TNFR1 and TNFR2 have shown that the two receptors share little homology in their intracellular domains and appear to activate distinct intracellular pathways [Tracey, 1994 #7415].

Recent studies with transgenic TNFR1 knockout mice have demonstrated that signalling through TNFR1 plays an important role in the clearing of low-level bacterial infection as well as TNF-induced septic shock following high-level bacterial infection [Lotz, 1996 #7436]. These findings indicate that compositions of matter which can inhibit signalling through the TNFR1 receptor may serve as useful targets for inhibition of TNF induced toxicities such as septic shock.

Antisense oligonucleotide inhibition of TNFR1 has proven to be a useful tool in understanding the role of TNFR1 stimulation in cytokine induction and cell proliferation. Ojwang et. al. have disclosed partial phosphothioate antisense oligodeoxynucleotides containing C-

-3-

5 propynyl or hexynyl derivatives of 2'-deoxyuridine which caused attenuation of TNFR1 mRNA and protein and inhibited TNF-alpha induced expression of IL-6 in MRC-5 cells [Ojwang, 1997 #5863]. These oligonucleotides were targeted to the poly (A) signal site of TNFR1 mRNA. A uniform phosphorothioate oligonucleotide targeted to the translation initiation codon of TNFR1 was found to have no effect.

There remains a long-felt need for improved compositions and methods for inhibiting TNFR1 gene expression

SUMMARY OF THE INVENTION

The present invention is directed to antisense compounds, particularly oligonucleotides, which are targeted to a nucleic acid encoding TNFR1, and which modulate the expression of TNFR1. Pharmaceutical and other compositions comprising the antisense compounds of the invention are also provided. Further provided are methods of modulating the expression of TNFR1 in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of TNFR1 by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric antisense compounds, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding TNFR1, ultimately modulating the amount of TNFR1 produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding TNFR1. As used herein, the terms "target nucleic

-4-

acid" and "nucleic acid encoding TNFR1" encompass DNA encoding TNFR1, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense." The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of TNFR1. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding TNFR1. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur

-5-

such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding TNFR1, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e.,

-6-

5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene) and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene). The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target

-7-

regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds.

"Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense

-8-

compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, or in the case of in vitro assays, under conditions in which the assays are performed.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or

-9-

mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 30 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (i.e. from about 8 to about 30 linked nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as

-10-

forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111;

-11-

5,563,253; 5,571,799; 5,587,361; 5,625,050; and 5,697,248, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is

-12-

referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-\text{CH}_2-\text{NH}-\text{O}-\text{CH}_2-$, $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{O}-\text{CH}_2-$ [known as a methylene (methyylimino) or MMI backbone], $-\text{CH}_2-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-$, $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)-\text{CH}_2-$ and $-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-$ [wherein the native phosphodiester backbone is represented as $-\text{O}-\text{P}-\text{O}-\text{CH}_2-$] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl, O-, S-, or N-alkenyl, O, S- or N-alkynyl, or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_1 to C_{10} alkenyl and alkynyl. Particularly preferred are $\text{O}[(\text{CH}_2)_n\text{O}]_m\text{CH}_3$, $\text{O}(\text{CH}_2)_n\text{OCH}_3$, $\text{O}(\text{CH}_2)_n\text{NH}_2$, $\text{O}(\text{CH}_2)_n\text{CH}_3$, $\text{O}(\text{CH}_2)_n\text{ONH}_2$, and $\text{O}(\text{CH}_2)_n\text{ON}[(\text{CH}_2)_n\text{CH}_3]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl,

-13-

substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, **1995**, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in United States patent application Serial Number 09/016,520, filed on January 30, 1998, which is commonly owned with the instant application and the contents of which are herein incorporated by reference.

Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,0531; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant

-14-

application, and each of which is herein incorporated by reference, and allowed United States patent application 08/468,037, filed on June 5, 1995, United States patent X,XXX,XXX, which is commonly owned with the instant application and is also herein incorporated by reference.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, **1991**, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**. Certain of these nucleobases are particularly useful for

-15-

increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and allowed United States patent application 08/762,488, filed on December 10, **1996**, United States patent X,XXX,XXX, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, **1994**, 4, 1053-1060), a thioether, e.g., hexyl-

-16-

S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, **1993**, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, 259, 327-330; Svinarchuk et al., *Biochimie*, **1993**, 75, 49-54), a phospholipid, e.g., dihexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, 14, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxcholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, 277, 923-937).

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the

-17-

instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds.

"Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more

-18-

oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and allowed United States patent application serial number 08/465,880, filed on June 6, 1995, United States patent X,XXX,XXX, which is commonly owned with the instant application and also herein incorporated by reference.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative

-19-

United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.:

5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291;
5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330;
4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170;
5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978;
5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259;
5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of

which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

-20-

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are

5 N,N'-dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the

10 free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As

15 used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates,

20 salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with

25 inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic

30 acid, tartaric acid, lactic acid, oxalic acid, gluconic

35

-21-

acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or
5 isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid,
10 ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic
15 compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth,
20 ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium,
25 potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids
30 such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid,
35 p-toluenesulfonic acid, naphthalenedisulfonic acid,

-22-

polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of TNFR1 is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding TNFR1, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding TNFR1 can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of TNFR1 in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may

-23-

be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions and/or formulations comprising the oligonucleotides of the present invention may also include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, i.e., fatty acids, bile salts,

-24-

chelating agents, surfactants and non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, 8, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33). One or more

5 penetration enhancers from one or more of these broad categories may be included. Penetration enhancers are described in pending United States patent application 08/886,829, filed on July 1, 1997, United States patent X,XXX,XXX, and pending United States patent application

10 08/961,469, filed on October 31, 1997, United States patent X,XXX,XXX, both of which are commonly owned with the instant application and both of which are herein incorporated by reference.

Various fatty acids and their derivatives which act as

15 penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, ricinoleate, monoolein (a.k.a. 1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid,

20 glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically acceptable salts thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in*

25 *Therapeutic Drug Carrier Systems*, **1991**, 8:2, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7:1, 1-33; El-Hariri et al., *J. Pharm. Pharmacol.*, **1992**, 44, 651-654). Examples of some presently preferred fatty acids are sodium caprate and sodium

30 laurate, used singly or in combination at concentrations of 0.5 to 5%.

Preferred penetration enhancers are disclosed in pending United States patent application 08/886,829, filed on July 1, 1997, United States patent X,XXX,XXX, which is

-25-

commonly owned with the instant application and which is herein incorporated by reference.

The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, **1996**, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. Preferred bile salts are described in pending United States patent application 08/886,829, filed on July 1, 1997, United States patent X,XXX,XXX, which is commonly owned with the instant application and which is herein incorporated by reference. A presently preferred bile salt is chenodeoxycholic acid (CDCA) (Sigma Chemical Company, St. Louis, MO), generally used at concentrations of 0.5 to 2%.

Complex formulations comprising one or more penetration enhancers may be used. For example, bile salts may be used in combination with fatty acids to make complex formulations. Preferred combinations include CDCA combined with sodium caprate or sodium laurate (generally 0.5 to 5%).

Chelating agents include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, 8:2, 92-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7:1, 1-33; Buur et al., *J. Control Rel.*, **1990**, 14, 43-51).

-26-

Chelating agents have the added advantage of also serving as DNase inhibitors.

Surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and
5 polyoxyethylene-20-cetyl ether (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, 8:2, 92-191); and perfluorochemical emulsions, such as FC-43 (Takahashi et al., *J. Pharm. Pharmacol.*, **1988**, 40, 252-257).

Non-surfactants include, for example, unsaturated
10 cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, 8:2, 92-191); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J.*
15 *Pharm. Pharmacol.*, **1987**, 39, 621-626).

As used herein, "carrier compound" refers to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid by in vivo processes that reduce the
20 bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance,
25 can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially
30 phosphorothioated oligonucleotide in hepatic tissue is reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., *Antisense Res. Dev.*, **1995**, 5, 115-121; Takakura et al.,

-27-

Antisense & Nucl. Acid Drug Dev., **1996**, 6, 177-183).

In contrast to a carrier compound, a "pharmaceutically acceptable carrier" (excipient) is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The pharmaceutically acceptable carrier may be liquid or solid and is selected with the planned manner of administration in mind so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutically acceptable carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrates (e.g., starch, sodium starch glycolate, etc.); or wetting agents (e.g., sodium lauryl sulphate, etc.). Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are described in U.S. Patents Nos. 4,704,295; 4,556,552; 4,309,406; and 4,309,404.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceutically-active materials such as, e.g., antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional

-28-

materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the antisense compounds of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the in vivo stability of the compounds and/or to target the compounds to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layer(s) made up of lipids arranged in a bilayer configuration (see, generally, Chonn et al., *Current Op. Biotech.*, **1995**, 6, 698-708). Liposome preparation is described in pending United States patent application 08/961,469, filed on October 31, 1997, United States patent X,XXX,XXX, which is commonly owned with the instant application and which is herein incorporated by reference.

Certain embodiments of the invention provide for liposomes and other compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include, but are not limited to, anticancer drugs such as daunorubicin, dactinomycin, doxorubicin, bleomycin,

-29-

mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil (5-FU), floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, 5 vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 1206-1228). Antiinflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Examples of antisense oligonucleotides include, but are not limited to, those directed to the following targets as disclosed in the indicated U.S. Patents, or pending U.S. applications, which are commonly owned with the instant application and are hereby incorporated by reference, or the indicated published PCT applications: raf (WO 96/39415, WO 95/32987 and U.S. Patent Nos. 5,563,255, issued October 8, 1996, and 5,656,612, issued August 12, 1997), the p120 nucleolar antigen (WO 93/17125 and U.S. Patent No. 5,656,743, issued August 12, 1997), protein kinase C (WO 95/02069, WO 95/03833 and WO 93/19203), multidrug resistance-associated protein (WO 95/10938 and U.S. Patent No. 5,510,239, issued

-30-

March 23, 1996), subunits of transcription factor AP-1 (pending application U.S. Serial No. 08/837,201, filed April 14, 1997), Jun kinases (pending application U.S. Serial No. 08/910,629, filed August 13, 1997), MDR-1 (multidrug resistance glycoprotein; pending application U.S. Serial No. 08/731,199, filed September 30, 1997), HIV (U.S. Patent Nos. 5,166,195, issued November 24, 1992 and 5,591,600, issued January 7, 1997), herpesvirus (U.S. Patent No. 5,248,670, issued September 28, 1993 and U.S. Patent No. 5,514,577, issued May 7, 1996), cytomegalovirus (U.S. Patents 5,442,049, issued August 15, 1995 and 5,591,720, issued January 7, 1997), papillomavirus (U.S. Patent 5,457,189, issued October 10, 1995), intercellular adhesion molecule-1 (ICAM-1) (U.S. Patent 5,514,788, issued May 7, 1996), 5-lipoxygenase (U.S. Patent 5,530,114, issued June 25, 1996) and influenzavirus (U.S. Patent 5,580,767, issued December 3, 1996). Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50} s found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 μ g to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years.

-31-

Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 :g to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

EXAMPLES

Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, **1993**, 21, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes,

-32-

Needham MA).

2'-Fluoro amidites

2'-Fluorodeoxyadenosine amidites

2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, **1993**, 36, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl- (DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was

-33-

accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, **1995**, 78, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the

-34-

structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH₂Cl₂/acetone/MeOH (20:5:3) containing 0.5% Et₃NH. The residue was dissolved in CH₂Cl₂ (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70 product. The solvent was evaporated and triturated with CH₃CN (200 mL). The residue was dissolved in CHCl₃ (1.5 L) and extracted with 2x500 mL of saturated NaHCO₃ and 2x500 mL

-35-

of saturated NaCl. The organic phase was dried over Na₂SO₄, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et₃NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl₃ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl₃. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH₃CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH₃CN (1 L), cooled to

-36-

-5°C and stirred for 0.5 h using an overhead stirrer. POCl₃ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO₃ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH₄OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH₃ gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the

-37-

reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl_3 (700 mL) and extracted with saturated NaHCO_3 (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO_4 and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO_3 (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over MgSO_4 and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

2'-(Aminooxyethyl) nucleoside amidites and 2'-(dimethylaminooxyethyl) nucleoside amidites

Aminooxyethyl and dimethylaminooxyethyl amidites are prepared as per the methods of United States patent applications serial number 10/037,143, filed February 14, 1998, and serial number 09/016,520, filed January 30, 1998, each of which is commonly owned with the instant application and is herein incorporated by reference.

Example 2**Oligonucleotide synthesis**

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

-39-

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

5 Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

10 Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedi-methylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligo-
15 nucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are
20 herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

25 Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

PNA Synthesis

30 Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, **1996**, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262,

-40-

herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed
5 oligonucleotides/oligonucleosides of the invention can be
of several different types. These include a first type
wherein the "gap" segment of linked nucleosides is
positioned between 5' and 3' "wing" segments of linked
nucleosides and a second "open end" type wherein the "gap"
10 segment is located at either the 3' or the 5' terminus of
the oligomeric compound. Oligonucleotides of the first type
are also known in the art as "gapmers" or gapped
oligonucleotides. Oligonucleotides of the second type are
also known in the art as "hemimers" or "wingmers."

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric

Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl
phosphorothioate and 2'-deoxy phosphorothioate oligo-
nucleotide segments are synthesized using an Applied
20 Biosystems automated DNA synthesizer Model 380B, as above.
Oligonucleotides are synthesized using the automated
synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphor-
amidite for the DNA portion and 5'-dimethoxytrityl-2'-O-
methyl-3'-O-phosphoramidite for 5' and 3' wings. The
25 standard synthesis cycle is modified by increasing the wait
step after the delivery of tetrazole and base to 600 s
repeated four times for RNA and twice for 2'-O-methyl. The
fully protected oligonucleotide is cleaved from the support
and the phosphate group is deprotected in 3:1
30 Ammonia/Ethanol at room temperature overnight then
lyophilized to dryness. Treatment in methanolic ammonia
for 24 hrs at room temperature is then done to deprotect
all bases and sample was again lyophilized to dryness. The
pellet is resuspended in 1M TBAF in THF for 24 hrs at room
35 temperature to deprotect the 2' positions. The reaction is

-41-

then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometer.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,4-dithiolane-2-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass column

-42-

(Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ³¹P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, 266, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,4-dihydro-2H-benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyl-diisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH₄OH at elevated temperature (55-60°C) for 12-16 hours and the released product then

-43-

dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

5 **Example 8**

Oligonucleotide Analysis - 96 Well Plate Format

 The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in
10 either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds
15 utilizing Electrospray-Mass Spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

20 **Example 9**

Cell culture and oligonucleotide treatment

 The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at
25 measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following four cell types are provided for illustrative purposes, but other cell types can be routinely used.

 T-24 cells:

30 The transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal
35 calf serum (Gibco/Life Technologies, Gaithersburg, MD),

-44-

penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

-45-

Treatment with antisense compounds:

When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 μ L OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 μ L of OPTI-MEM™-1 containing 3.75 μ g/mL LIPOFECTIN™ (Gibco BRL) and the desired oligonucleotide at a final concentration of 150 nM. After 4 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16 hours after oligonucleotide treatment.

Example 10

Antisense inhibition of TNFR1 expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human TNFR1 RNA, using published sequences (GenBank accession number X55313, incorporated herein as SEQ ID NO: 1). The oligonucleotides are shown in Table 1. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. X55313), to which the oligonucleotide binds. All compounds in Table 1 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on TNFR1 mRNA levels by quantitative real-time PCR as described in subsequent examples. Data are averages from three experiments.

Table 1

Inhibition of TNFR1 mRNA levels by phosphorothioate oligodeoxynucleotides

| ISIS# | REGION | TARGET SITE | SEQUENCE | % Inhibition | SEQ ID NO. |
|-------|--------|-------------|--------------------|--------------|------------|
| 18875 | 5' UTR | 37 | TTCTCTGGACTGAGGCTC | 19 | 8 |
| 18876 | 5' UTR | 68 | TCCCCTCCTCTCTGCTTT | 5 | 9 |
| 18877 | 5' UTR | 109 | AGACTCGGGCATAGAGAT | 0 | 10 |
| 18878 | 5' UTR | 114 | GGTTGAGACTCGGGCATA | 40 | 11 |
| 18879 | 5' UTR | 118 | TGAGGGTTGAGACTCGGG | 2 | 12 |
| 18880 | 5' UTR | 123 | ACAGTTGAGGGTTGAGAC | 30 | 13 |

-46-

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|----|-------|--------|------|--------------------|----|----|
| | 18881 | 5' UTR | 127 | GGTGACAGTTGAGGGTTG | 8 | 14 |
| | 18882 | 5' UTR | 196 | GCAGTGTGGCAGCGGCAG | 53 | 15 |
| | 18883 | 5' UTR | 199 | AGGGCAGTGTGGCAGCGG | 53 | 16 |
| | 18884 | 5' UTR | 202 | CTCAGGGCAGTGTGGCAG | 61 | 17 |
| 5 | 18885 | 5' UTR | 207 | TTGGGCTCAGGGCAGTGT | 0 | 18 |
| | 18886 | 5' UTR | 210 | CATTTGGGCTCAGGGCAG | 9 | 19 |
| | 18887 | Coding | 262 | GTCAGGCACGGTGGAGAG | 0 | 20 |
| | 18888 | Coding | 266 | GCAGGTCAGGCACGGTGG | 16 | 21 |
| | 18889 | Coding | 272 | GCAGCAGCAGGTCAGGCA | 37 | 22 |
| 10 | 18890 | Coding | 276 | AGCGGCAGCAGCAGGTCA | 0 | 23 |
| | 18891 | Coding | 280 | CACCAGCGGCAGCAGCAG | 21 | 24 |
| | 18892 | Coding | 286 | CAGGAGCACCAGCGGCAG | 46 | 25 |
| | 18893 | Coding | 306 | TATATTCCCAACACAGC | 25 | 26 |
| | 18894 | Coding | 356 | TCTTCTCCCTGTCCCCTA | 13 | 27 |
| 15 | 18895 | Coding | 403 | ATTATTTTGAGGGTGGAT | 0 | 28 |
| | 18896 | Coding | 435 | GTTCTTTGTGGCACTTG | 12 | 29 |
| | 18897 | Coding | 440 | AGTAGGTTCTTTGTGGC | 46 | 30 |
| | 18898 | Coding | 460 | GCCTGGACAGTCATTGTA | 0 | 31 |
| | 18899 | Coding | 480 | CAGTCCGTATCCTGCCCC | 26 | 32 |
| 20 | 18900 | Coding | 500 | AGCCGCTCTCACACTCCC | 36 | 33 |
| | 18901 | Coding | 516 | TCTGAAGCGGTGAAGGAG | 0 | 34 |
| | 18902 | Coding | 521 | GGTTTTCTGAAGCGGTGA | 17 | 35 |
| | 18903 | Coding | 525 | AGGTGGTTTTCTGAAGCG | 0 | 36 |
| | 18904 | Coding | 530 | GTCTGAGGTGGTTTTCTG | 34 | 37 |
| 25 | 18905 | Coding | 537 | AGGCAGTGTCTGAGGTGG | 0 | 38 |
| | 18906 | Coding | 542 | AGCTGAGGCAGTGTCTGA | 27 | 39 |
| | 18907 | Coding | 565 | CATTTCTTTTCGGCATT | 13 | 40 |
| | 18908 | Coding | 569 | GACCCATTTCTTTTCGGC | 26 | 41 |
| | 18909 | Coding | 574 | CACCTGACCCATTTCTTT | 46 | 42 |
| 30 | 18910 | Coding | 635 | GGTACTGGTTCTTCTTGC | 14 | 43 |
| | 18911 | Coding | 654 | TTTTCACTCCAATAATGC | 0 | 44 |
| | 18912 | Coding | 693 | CCATTGAGGCAGAGGCTG | 48 | 45 |
| | 18913 | Coding | 699 | ACGGTCCCATTGAGGCAG | 34 | 46 |
| | 18914 | Coding | 732 | ACGGTGTTCTGTTTCTCC | 7 | 47 |
| 35 | 18915 | Coding | 786 | CTACAGGAGACACACTCG | 28 | 48 |
| | 18916 | Coding | 796 | CTTACAGTTACTACAGGA | 21 | 49 |
| | 18917 | Coding | 802 | GCTTTTCTTACAGTTACT | 10 | 50 |
| | 18918 | Coding | 807 | TCCAGGCTTTTCTTACAG | 0 | 51 |
| | 18919 | Coding | 845 | TAACATTCTCAATCTGGG | 0 | 52 |
| 40 | 18920 | Coding | 873 | ACTGTGGTGCCTGAGTCC | 31 | 53 |
| | 18921 | Coding | 906 | CAAAGACCAAAGAAAATG | 29 | 54 |
| | 18922 | Coding | 911 | AAAGGCAAAGACCAAAGA | 31 | 55 |
| | 18923 | Coding | 921 | AGGAGGGATAAAAGGCAA | 22 | 56 |
| | 18924 | Coding | 929 | CAATGAAGAGGAGGGATA | 21 | 57 |
| 45 | 18925 | Coding | 935 | TTAAACCAATGAAGAGGA | 28 | 58 |
| | 18926 | Coding | 952 | CCGTTGGTAGCGATACAT | 30 | 59 |
| | 18927 | Coding | 992 | TCGATTTCCCAACAAACA | 1 | 60 |
| | 18928 | Coding | 1033 | CTTAGTAGTAGTTCCTTC | 15 | 61 |
| | 18929 | Coding | 1075 | GAAGCCTGGAGTGGGACT | 48 | 62 |
| 50 | 18930 | Coding | 1098 | GGACTGAAGCCCAGGGTG | 12 | 63 |
| | 18931 | Coding | 1113 | GTGGAAGTGGGCACGGGA | 4 | 64 |
| | 18932 | Coding | 1118 | TGAAGGTGGAAGTGGGCA | 27 | 65 |

-47-

| | | | | | | |
|----|-------|--------|------|---------------------|----|----|
| | 18933 | Coding | 1127 | AGCTGGAGGTGAAGGTGG | 0 | 66 |
| | 18934 | Coding | 1162 | CGCAAAGTTGGGACAGTC | 30 | 67 |
| | 18935 | Coding | 1184 | GTGCCACCTCTCTGCGGG | 0 | 68 |
| | 18936 | Coding | 1269 | CTGTCCTCCCACCTTCTGA | 16 | 69 |
| 5 | 18937 | Coding | 1290 | AGGCTCTGTGGCTTGTGG | 47 | 70 |
| | 18938 | Coding | 1389 | TCGTGGTTCGCTCAGCCCT | 28 | 71 |
| | 18939 | Coding | 1465 | CCGCCTCCAGGTCGCCAG | 0 | 72 |
| | 18940 | Coding | 1537 | GCAGCCCAGCAGGTCCAT | 32 | 73 |
| | 18941 | Coding | 1545 | TCCTCCAGGCAGCCCAGC | 41 | 74 |
| 10 | 18942 | Coding | 1604 | ATCTGAGAAGACTGGGCG | 0 | 75 |
| | 18943 | Coding | 1707 | GCTCCTGCTTGCCCCCTGC | 43 | 76 |
| | 18944 | Coding | 1732 | GTTAGCACCAAGTAGGCG | 11 | 77 |
| | 18945 | Coding | 1842 | CGCAAACCACCCACTCAG | 51 | 78 |
| | 18946 | Coding | 1847 | ATCCTCGCAAACCACCCA | 29 | 79 |
| 15 | 18947 | Coding | 1859 | ATAGCGTCCCTCATCCTC | 34 | 80 |
| | 18948 | Coding | 1925 | CTCAGGGACGAACCAGGG | 3 | 81 |
| | 18949 | Coding | 1930 | AAAGGCTCAGGGACGAAC | 42 | 82 |
| | 18950 | Coding | 1979 | ACAAAACAAAACAAAACA | 27 | 83 |
| | 18951 | Coding | 2016 | GCCAAGTTTCTATTAGTG | 10 | 84 |
| 20 | 18952 | Coding | 2033 | GCAGAGGGCAGAGGAGTG | 24 | 85 |
| | 18953 | Coding | 2039 | GTCCAGGCAGAGGGCACA | 21 | 86 |
| | 18954 | Coding | 2043 | GCTTGTCCAGGCAGAGGG | 18 | 87 |
| | 18955 | Coding | 2071 | TGCCTTAGGACAGTTCAG | 20 | 88 |
| | 18956 | Coding | 2085 | TCCGTGCTCGCCCCCTGCC | 19 | 89 |
| 25 | 18957 | Coding | 2089 | TTGTTCCGTGCTCGCCCC | 41 | 90 |
| | 18958 | Coding | 2097 | AGGCCCCATTGTTCCGTG | 0 | 91 |

As shown in Table 1, SEQ ID NOs 11, 15, 16, 17, 22, 25, 30, 33, 42, 45, 62, 70, 74, 76, 78, 82 and 90

30 demonstrated at least 35% inhibition of TNFR1 expression in this assay and are therefore preferred.

Example 11

Analysis of oligonucleotide inhibition of TNFR1 expression

Antisense modulation of TNFR1 expression can be assayed
 35 in a variety of ways known in the art. For example, TNFR1 mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total
 40 cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Northern

-48-

blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., **1996**. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions. Other methods of PCR are also known in the art.

TNFR1 protein levels can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to TNFR1 can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., **1997**. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., **1997**.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., **1998**. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., **1997**. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current*

-49-

Protocols in Molecular Biology, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

Example 12**Poly(A)+ mRNA isolation**

5 Poly(A)+ mRNA was isolated according to Miura et al.,
Clin. Chem., 1996, 42, 1758-1764. Other methods for
poly(A)+ mRNA isolation are taught in, for example,
Ausubel, F.M. et al., *Current Protocols in Molecular*
Biology, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons,
10 Inc., 1993. Briefly, for cells grown on 96-well plates,
growth medium was removed from the cells and each well was
washed with 200 μ L cold PBS. 60 μ L lysis buffer (10 mM
Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM
vanadyl-ribonucleoside complex) was added to each well, the
15 plate was gently agitated and then incubated at room
temperature for five minutes. 55 μ L of lysate was
transferred to Oligo d(T) coated 96-well plates (AGCT Inc.,
Irvine CA). Plates were incubated for 60 minutes at room
temperature, washed 3 times with 200 μ L of wash buffer (10
20 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final
wash, the plate was blotted on paper towels to remove
excess wash buffer and then air-dried for 5 minutes. 60 μ L
of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C
was added to each well, the plate was incubated on a 90° hot
25 plate for 5 minutes, and the eluate was then transferred to
a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be
treated similarly, using appropriate volumes of all
solutions.

30 Example 13**Total RNA Isolation**

Total mRNA was isolated using an RNEASY 96™ kit and
buffers purchased from Qiagen Inc. (Valencia CA) following
the manufacturer's recommended procedures. Briefly, for

-50-

cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold PBS. 100 μ L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 μ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 μ L water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 μ L water.

Example 14**Real-time Quantitative PCR Analysis of TNFR1 mRNA Levels**

Quantitation of TNFR1 mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an

-51-

oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE or FAM, PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular (six-second) intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM MgCl₂, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 µL poly(A) mRNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. following a 10 minute incubation at 95°C to activate the AMPLITAQ

-52-

GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

For TNFR1 the PCR primers were:

5 forward primer: GCTTCAGAAAACACCTCAGACA (SEQ ID No. 2)
reverse primer: CCGGTCCACTGTGCAAGAA (SEQ ID No. 3) and the
PCR probe was: FAM-TCAGCTGCTCCAAATGCCGAAAGG-TAMRA
(SEQ ID No. 4) where FAM (PE-Applied Biosystems, Foster
City, CA) is the fluorescent reporter dye) and TAMRA (PE-
10 Applied Biosystems, Foster City, CA) is the quencher dye.

For GAPDH the PCR primers were:

forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID No. 5)
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID No. 6) and the
PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ
15 ID No. 7) where JOE (PE-Applied Biosystems, Foster City,
CA) is the fluorescent reporter dye) and TAMRA (PE-Applied
Biosystems, Foster City, CA) is the quencher dye.

Example 15

Northern blot analysis of TNFR1 mRNA levels

20 Eighteen hours after antisense treatment, cell
monolayers were washed twice with cold PBS and lysed in 1
mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA
was prepared following manufacturer's recommended
protocols. Twenty micrograms of total RNA was fractionated
25 by electrophoresis through 1.2% agarose gels containing
1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc.
Solon, OH). RNA was transferred from the gel to HYBOND™-N+
nylon membranes (Amersham Pharmacia Biotech, Piscataway,
NJ) by overnight capillary transfer using a
30 Northern/Southern Transfer buffer system (TEL-TEST "B"
Inc., Friendswood, TX). RNA transfer was confirmed by UV
visualization. Membranes were fixed by UV cross-linking
using a STRATALINKER™ UV Crosslinker 2400 (Stratagene,
Inc, La Jolla, CA).

35 Membranes were probed using QUICKHYB™ hybridization

-53-

solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions with a TNFR1 specific probe prepared by PCR using the forward primer GCTTCAGAAAACACCTCAGACA (SEQ ID No. 2) and the reverse primer CCGGTCCACTGTGCAAGAA (SEQ ID No. 3). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) RNA (Clontech, Palo Alto, CA). Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to G3PDH levels in untreated controls.

Example 16**Western blot analysis of TNFR1 protein levels**

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 hr after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100μl/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to TNFR1 is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

Example 17:**Antisense inhibition of TNFR1 expression- phosphorothioate 2'-MOE gapmer oligonucleotides**

In accordance with the present invention, a second series of oligonucleotides targeted to human TNFR1 were synthesized. The oligonucleotide sequences are shown in Table 2. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. X55313), to which the oligonucleotide binds.

-54-

All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings." The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in previous examples and are averaged from three experiments.

Table 2

Inhibition of TNFR1 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

| | ISIS# | REGION | TARGET SITE | SEQUENCE | % Inhibition | SEQ ID NO. |
|----|-------|--------|----------------|--------------------|-----------------|---------------|
| | | | | | | |
| 20 | 19463 | 5' UTR | 37 | TTCTCTGGACTGAGGCTC | 72 | 8 |
| | 19464 | 5' UTR | 68 | TCCCCTCCTCTCTGCTTT | 9 | 9 |
| | 19465 | 5' UTR | 109 | AGACTCGGGCATAGAGAT | 18 | 10 |
| | 19466 | 5' UTR | 114 | GGTTGAGACTCGGGCATA | 95 | 11 |
| | 19467 | 5' UTR | 118 | TGAGGGTTGAGACTCGGG | 28 | 12 |
| 25 | 19468 | 5' UTR | 123 | ACAGTTGAGGGTTGAGAC | 66 | 13 |
| | 19469 | 5' UTR | 127 | GGTGACAGTTGAGGGTTG | 42 | 14 |
| | 19470 | 5' UTR | 196 | GCAGTGTGGCAGCGGCAG | 78 | 15 |
| | 19471 | 5' UTR | 199 | AGGGCAGTGTGGCAGCGG | 76 | 16 |
| | 19472 | 5' UTR | 202 | CTCAGGGCAGTGTGGCAG | 90 | 17 |
| 30 | 19473 | 5' UTR | 207 | TTGGGCTCAGGGCAGTGT | 48 | 18 |
| | 19474 | 5' UTR | 210 | CATTTGGGCTCAGGGCAG | 70 | 19 |
| | 19475 | Coding | 262 | GTCAGGCACGGTGGAGAG | 66 | 20 |
| | 19476 | Coding | 266 | GCAGGTCAGGCACGGTGG | 91 | 21 |
| | 19477 | Coding | 272 | GCAGCAGCAGGTCAGGCA | 85 | 22 |
| 35 | 19478 | Coding | 276 | AGCGGCAGCAGCAGGTCA | 93 | 23 |
| | 19479 | Coding | 280 | CACCAGCGGCAGCAGCAG | 65 | 24 |
| | 19480 | Coding | 286 | CAGGAGCACCAGCGGCAG | 60 | 25 |
| | 19481 | Coding | 306 | TATATTCCCAACACAGC | 58 | 26 |
| | 19482 | Coding | 356 | TCTTCTCCCTGTCCCCTA | 42 | 27 |
| 40 | 19483 | Coding | 403 | ATTATTTTGAGGGTGGAT | 75 | 28 |
| | 19484 | Coding | 435 | GTTCCTTTGTGGCACTTG | 88 | 29 |
| | 19485 | Coding | 440 | AGTAGGTTCCTTTGTGGC | 78 | 30 |
| | 19486 | Coding | 460 | GCCTGGACAGTCATTGTA | 80 | 31 |
| | 19487 | Coding | 480 | CAGTCCGTATCCTGCCCC | 66 | 32 |
| | 19488 | Coding | 500 | AGCCGCTCTCACACTCCC | 86 | 33 |

-55-

| | | | | | | |
|----|-------|--------|------|--------------------|----|----|
| | 19489 | Coding | 516 | TCTGAAGCGGTGAAGGAG | 52 | 34 |
| | 19490 | Coding | 521 | GGTTTTCTGAAGCGGTGA | 92 | 35 |
| | 19491 | Coding | 525 | AGGTGGTTTTCTGAAGCG | 82 | 36 |
| | 19492 | Coding | 530 | GTCTGAGGTGGTTTTCTG | 91 | 37 |
| 5 | 19493 | Coding | 537 | AGGCAGTGTCTGAGGTGG | 96 | 38 |
| | 19494 | Coding | 542 | AGCTGAGGCAGTGTCTGA | 79 | 39 |
| | 19495 | Coding | 565 | CATTTCTTTTCGGCATT | 41 | 40 |
| | 19496 | Coding | 569 | GACCCATTTCTTTTCGGC | 93 | 41 |
| | 19497 | Coding | 574 | CACCTGACCCATTTCTT | 63 | 42 |
| 10 | 19498 | Coding | 635 | GGTACTGGTTCTTCTG | 79 | 43 |
| | 19499 | Coding | 654 | TTTTCACTCCAATAATGC | 9 | 44 |
| | 19500 | Coding | 693 | CCATTGAGGCAGAGGCTG | 0 | 45 |
| | 19501 | Coding | 699 | ACGGTCCCATTGAGGCAG | 81 | 46 |
| | 19502 | Coding | 732 | ACGGTGTCTGTTTCTCC | 77 | 47 |
| 15 | 19503 | Coding | 786 | CTACAGGAGACACACTCG | 81 | 48 |
| | 19504 | Coding | 796 | CTTACAGTTACTACAGGA | 61 | 49 |
| | 19505 | Coding | 802 | GCTTTTCTTACAGTTACT | 93 | 50 |
| | 19506 | Coding | 807 | TCCAGGCTTTTCTTACAG | 71 | 51 |
| | 19507 | Coding | 845 | TAACATTCTCAATCTGGG | 0 | 52 |
| 20 | 19508 | Coding | 873 | ACTGTGGTGCCTGAGTCC | 74 | 53 |
| | 19509 | Coding | 906 | CAAAGACCAAAGAAAATG | 29 | 54 |
| | 19510 | Coding | 911 | AAAGGCAAAGACCAAAGA | 45 | 55 |
| | 19511 | Coding | 921 | AGGAGGGATAAAAGGCAA | 67 | 56 |
| | 19512 | Coding | 929 | CAATGAAGAGGAGGGATA | 55 | 57 |
| 25 | 19513 | Coding | 935 | TTAAACCAATGAAGAGGA | 25 | 58 |
| | 19514 | Coding | 952 | CCGTTGGTAGCGATACAT | 93 | 59 |
| | 19515 | Coding | 992 | TCGATTTCCCAACAA | 16 | 60 |
| | 19516 | Coding | 1033 | CTTAGTAGTAGTTCCTTC | 70 | 61 |
| | 19517 | Coding | 1075 | GAAGCCTGGAGTGGGACT | 0 | 62 |
| 30 | 19518 | Coding | 1098 | GGACTGAAGCCCAGGGTG | 31 | 63 |
| | 19519 | Coding | 1113 | GTGGAAGTGGGCACGGGA | 41 | 64 |
| | 19520 | Coding | 1118 | TGAAGGTGGAAGTGGGCA | 51 | 65 |
| | 19521 | Coding | 1127 | AGCTGGAGGTGAAGGTGG | 59 | 66 |
| | 19522 | Coding | 1162 | CGCAAAGTTGGGACAGTC | 80 | 67 |
| 35 | 19523 | Coding | 1184 | GTGCCACCTCTCTGCGGG | 40 | 68 |
| | 19524 | Coding | 1269 | CTGTCTCTCCACTTCTGA | 67 | 69 |
| | 19525 | Coding | 1290 | AGGCTCTGTGGCTTGTGG | 79 | 70 |
| | 19526 | Coding | 1389 | TCGTGGTCGCTCAGCCCT | 75 | 71 |
| | 19527 | Coding | 1465 | CCGCCTCCAGGTCGCCAG | 57 | 72 |
| 40 | 19528 | Coding | 1537 | GCAGCCCAGCAGGTCCAT | 68 | 73 |
| | 19529 | Coding | 1545 | TCCTCCAGGCAGCCAGC | 80 | 74 |
| | 19530 | Coding | 1604 | ATCTGAGAAGACTGGGCG | 19 | 75 |
| | 19531 | Coding | 1707 | GCTCCTGCTTGCCCCTGC | 89 | 76 |
| | 19532 | Coding | 1732 | GTTAGCACCAAGTAGGCG | 80 | 77 |
| 45 | 19533 | Coding | 1842 | CGCAAACCAACCACTCAG | 79 | 78 |
| | 19534 | Coding | 1847 | ATCCTCGCAAACCAACCA | 42 | 79 |
| | 19535 | Coding | 1859 | ATAGCGTCCCTCATCCTC | 52 | 80 |
| | 19536 | Coding | 1925 | CTCAGGGACGAACCAAGG | 92 | 81 |
| | 19537 | Coding | 1930 | AAAGGCTCAGGGACGAAC | 41 | 82 |
| 50 | 19538 | Coding | 1979 | ACAAAACAAAACAAAACA | 0 | 83 |
| | 19539 | Coding | 2016 | GCCAAGTTTCTATTAGTG | 87 | 84 |
| | 19540 | Coding | 2033 | GCAGAGGGCACAGGAGTG | 59 | 85 |

-56-

| | | | | | | |
|---|-------|--------|------|--------------------|----|----|
| 5 | 19541 | Coding | 2039 | GTCCAGGCAGAGGGCACA | 72 | 86 |
| | 19542 | Coding | 2043 | GCTTGTCCAGGCAGAGGG | 58 | 87 |
| | 19543 | Coding | 2071 | TGCCTTAGGACAGTTCAG | 69 | 88 |
| | 19544 | Coding | 2085 | TCCGTGCTCGCCCCTGCC | 62 | 89 |
| | 19545 | Coding | 2089 | TTGTTCCGTGCTCGCCCC | 57 | 90 |
| | 19546 | Coding | 2097 | AGGCCCCATTGTTCCGTG | 79 | 91 |

As shown in Table 2, SEQ ID NOs 11, 15, 16, 17, 21, 22, 23, 28, 29, 30, 31, 33, 35, 36, 37, 38, 39, 41, 43, 46, 47, 48, 50, 59, 67, 70, 71, 74, 76, 77, 78, 81, 84 and 91 demonstrated at least 75% inhibition of TNFR1 expression in this experiment and are therefore preferred.

-57-

What is claimed is:

1. An antisense compound 8 to 30 nucleotides in length targeted to a nucleic acid molecule encoding human TNFR1, wherein said antisense compound inhibits the expression of human TNFR1.
2. The antisense compound of claim 1 which is an antisense oligonucleotide.
3. The oligonucleotide of claim 2 comprising SEQ ID NO 11, 15, 16, 17, 21, 22, 23, 25, 28, 29, 30, 31, 33, 35, 36, 37, 38, 39, 41, 42, 43, 45, 46, 47, 48, 50, 59, 62, 67, 70, 71, 74, 76, 77, 78, 81, 82, 84, 90 and 91.
4. The oligonucleotide of claim 2 comprising SEQ ID NO: 11, 15, 16, 17, 22, 30, 33, 70, 74, 76 and 78.
5. The oligonucleotide of claim 2 which comprises at least one modified internucleoside linkage.
6. The oligonucleotide of claim 5 wherein the modified internucleoside linkage is a phosphorothioate linkage.
7. The oligonucleotide of claim 2 which comprises at least one modified sugar moiety.
8. The oligonucleotide of claim 7 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.
9. The oligonucleotide of claim 2 which comprises at least one modified nucleobase.
10. The oligonucleotide of claim 9 wherein the modified nucleobase is a 5-methylcytosine.
11. The oligonucleotide of claim 2 which is a chimeric oligonucleotide.
12. A pharmaceutical composition comprising the antisense compound of claim 1 and a pharmaceutically acceptable carrier or diluent.
13. The pharmaceutical composition of claim 12 comprising a colloidal dispersion system.
14. The pharmaceutical composition of claim 12 wherein the antisense compound is an antisense oligonucleotide.

-58-

15. A method of inhibiting the expression of TNFR1 in human cells or tissues comprising contacting said cells or tissues with the antisense compound of claim 1 so that expression of TNFR1 is inhibited.

5 16. A method of treating a human having a disease or condition associated with TNFR1 comprising administering to said animal a therapeutically or prophylactically effective amount of the antisense compound of claim 1 so that expression of TNFR1 is inhibited.

10 17. The method of claim 16 wherein the disease or condition is an inflammatory condition.

AMENDED CLAIMS

[received by the International Bureau on 18 November 1999 (18.11.99);
original claims 1, 3-11 and 13 amended; remaining claims unchanged (2 pages)]

5 1. An antisense compound 8 to 30 nucleobases in length
targeted to a nucleic acid molecule encoding human TNFR1,
wherein said antisense compound inhibits the expression of
human TNFR1.

 2. The antisense compound of claim 1 which is an
antisense oligonucleotide.

10 3. The antisense compound of claim 2 wherein the
antisense compound has a sequence comprising SEQ ID NO 11,
15, 16, 17, 21, 22, 23, 25, 28, 29, 30, 31, 33, 35, 36, 37,
38, 39, 41, 42, 43, 45, 46, 47, 48, 50, 59, 62, 67, 70, 71,
74, 76, 77, 78, 81, 82, 84, 90 or 91.

15 4. The antisense compound of claim 2 wherein the
antisense compound has a sequence comprising SEQ ID NO: 11,
15, 16, 17, 22, 30, 33, 70, 74, 76 or 78.

 5. The antisense compound of claim 2 wherein the
antisense oligonucleotide has a sequence comprising at least
one modified internucleoside linkage.

20 6. The antisense compound of claim 5 wherein the
modified internucleoside linkage is a phosphorothioate
linkage.

 7. The antisense compound of claim 2 wherein the
antisense oligonucleotide comprises at least one modified
sugar moiety.

25 8. The antisense compound of claim 7 wherein the
modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

 9. The antisense compound of claim 2 wherein the
antisense oligonucleotide comprises at least one modified
nucleobase.

30 10. The antisense compound of claim 9 wherein the
modified nucleobase is a 5-methylcytosine.

 11. The antisense compound of claim 2 wherein the
antisense oligonucleotide is a chimeric oligonucleotide.

35 12. A pharmaceutical composition comprising the antisense
compound of claim 1 and a pharmaceutically acceptable carrier

or diluent.

13. The pharmaceutical composition of claim 12 further comprising a colloidal dispersion system.

14. The pharmaceutical composition of claim 12 wherein
5 the antisense compound is an antisense oligonucleotide.

Statement Under Article 19(1)

This is in response to the International Search Report mailed 21 October 1999 setting a two (2) month period for reply.

Claims 3-11 have been amended to correct antecedent basis for the term "oligonucleotide". Claim 1 has been amended to more properly recite that the antisense compound comprises nucleobases as disclosed throughout the specification (for example, at page 9, lines 15-17). Claim 13 has been amended to make clear that the pharmaceutical composition may further comprise a colloidal dispersion system. No new matter is added by this amendment. Substitute sheets for pages 57 and 58 are enclosed.

Claims 2, 12 and 14-17 are unchanged.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Brenda F. Baker and Lex M. Cowsert

(ii) TITLE OF INVENTION: ANTISENSE MODULATION OF TNFR1 EXPRESSION

(iii) NUMBER OF SEQUENCES: 91

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(C) CITY: Marlton

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 inch disk, 1.44 Mb

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: Windows 95

(D) SOFTWARE: Word Perfect 6.1 for Windows

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: Not Yet Assigned

(B) FILING DATE: Herewith

(C) CLASSIFICATION: N/A

(viii) ATTORNEY/AGENT INFORMATION:

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(C) REFERENCE/DOCKET NUMBER: ISPH-0358

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(A) TELEPHONE: (609) 810-1515

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(2) INFORMATION FOR SEQ ID NO: 1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2161

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1

| | | | | | |
|-------------|------------|------------|------------|------------|------|
| CGGCCCAGTG | ATCTTGAACC | CCAAAGGCCA | GAACTGGAGC | CTCAGTCCAG | 50 |
| AGAATTCTGA | GAAAATTAAA | GCAGAGAGGA | GGGGAGAGAT | CACTGGGACC | 100 |
| AGGCCGTGAT | CTCTATGCCC | GAGTCTCAAC | CCTCAACTGT | CACCCCAAGG | 150 |
| CACTTGGGAC | GTCCTGGACA | GACCGAGTCC | CGGGAAGCCC | CAGCACTGCC | 200 |
| GCTGCCACAC | TGCCCTGAGC | CCAAATGGGG | GAGTGAGAGG | CCATAGCTGT | 250 |
| CTGGCATGGG | CCTCTCCACC | GTGCCTGACC | TGCTGCTGCC | GCTGGTGCTC | 300 |
| CTGGAGCTGT | TGGTGGGAAT | ATACCCCTCA | GGGGTTATTG | GACTGGTCCC | 350 |
| TCACCTAGGG | GACAGGGAGA | AGAGAGATAG | TGTGTGTCCC | CAAGGAAAAT | 400 |
| ATATCCACCC | TCAAAATAAT | TCGATTTGCT | GTACCAAGTG | CCACAAAGGA | 450 |
| ACCTACTTGT | ACAATGACTG | TCCAGGCCCC | GGGCAGGATA | CGGACTGCAG | 500 |
| GGAGTGTGAG | AGCGGCTCCT | TCACCGCTTC | AGAAAACCAC | CTCAGACACT | 550 |
| GCCTCAGCTG | CTCCAAATGC | CGAAAGGAAA | TGGGTCAGGT | GGAGATCTCT | 600 |
| TCTTGACACAG | TGGACCGGGA | CACCGTGTGT | GGCTGCAGGA | AGAACCAGTA | 650 |
| CCGGCATTAT | TGGAGTGAAA | ACCTTTTCCA | GTGCTTCAAT | TGCAGCCTCT | 700 |
| GCCTCAATGG | GACCGTGAC | CTCTCCTGCC | AGGAGAAACA | GAACACCGTG | 750 |
| TGCACCTGCC | ATGCAGGTTT | CTTTCTAAGA | GAAAACGAGT | GTGTCTCCTG | 800 |
| TAGTAACTGT | AAGAAAAGCC | TGGAGTGCAC | GAAGTTGTGC | CTACCCCAGA | 850 |
| TTGAGAATGT | TAAGGGCACT | GAGGACTCAG | GCACCACAGT | GCTGTTGCCC | 900 |
| CTGGTCATTT | TCTTTGGTCT | TTGCCTTTTA | TCCCTCCTCT | TCATTGGTTT | 950 |
| AATGTATCGC | TACCAACGGT | GGAAGTCCAA | GCTCTACTCC | ATTGTTTGTG | 1000 |
| GGAAATCGAC | ACCTGAAAAA | GAGGGGGAGC | TTGAAGGAAC | TACTACTAAG | 1050 |
| CCCCTGGCCC | CAAACCCAAG | CTTCAGTCCC | ACTCCAGGCT | TCACCCCCAC | 1100 |
| CCTGGGCTTC | AGTCCCGTGC | CCAGTTCCAC | CTTCACCTCC | AGCTCCACCT | 1150 |
| ATACCCCCGG | TGACTGTCCC | AACTTTGCGG | CTCCCCGCAG | AGAGGTGGCA | 1200 |
| CCACCCTATC | AGGGGGCTGA | CCCCATCCTT | GCGACAGCCC | TCGCCTCCGA | 1250 |
| CCCCATCCCC | AACCCCTTC | AGAAGTGGGA | GGACAGCGCC | CACAAGCCAC | 1300 |
| AGAGCCTAGA | CACTGATGAC | CCGCGACGC | TGTACGCCGT | GGTGGAGAAC | 1350 |
| GTGCCCCCGT | TGCGCTGGAA | GGAATTCGTG | CGGCGCCTAG | GGCTGAGCGA | 1400 |

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CCACGAGATC GATCGGCTGG AGCTGCAGAA CGGGCGCTGC CTGCGCGAGG 1450
CGCAATACAG CATGCTGGCG ACCTGGAGGC GCGGCACGCC GCGGCGCGAG 1500
GCCACGCTGG AGCTGCTGGG ACGCGTGCTC CGCGACATGG ACCTGCTGGG 1550
CTGCCTGGAG GACATCGAGG AGGCGCTTTG CGGCCCCGCC GCCCTCCCGC 1600
CCGCGCCCCAG TCTTCTCAGA TGAGGCTGCG CCCCTGCGGG CAGCTCTAAG 1650
GACCGTCCTG CGAGATCGCC TTCCAACCCC ACTTTTTTCT GGAAAGGAGG 1700
GGTCCTGCAG GGGCAAGCAG GAGCTAGCAG CCGCCTACTT GGTGCTAACC 1750
CCTCGATGTA CATAGCTTTT CTCAGCTGCC TGCGCGCCGC CGACAGTCAG 1800
CGCTGTGCGC GCGGAGAGAG GTGCGCCGTG GGCTCAAGAG CCTGAGTGGG 1850
TGGTTTGCGA GGATGAGGGA CGCTATGCCT CATGCCCCGT TTGGGTGTCC 1900
TCACCAGCAA GGCTGCTCGG GGGCCCCTGG TTCGTCCCTG AGCCTTTTTT 1950
ACAGTGCATA AGCAGTTTTT TTTGTTTTTG TTTTGTTTTG TTTTGTTTTT 2000
AAATCAATCA TGTTACACTA ATAGAACTT GGCACCTCTG TGCCCTCTGC 2050
CTGGACAAGC ACATAGCAAG CTGAACTGTC CTAAGGCAGG GCGGAGCACG 2100
GAACAATGGG GCCTTCAGCT GGAGCTGTGG ACTTTTGTAC ATACACTAAA 2150
ATTCTGAAGT T 2161

```

(2) INFORMATION FOR SEQ ID NO: 2

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2

GCTTCAGAAA ACCACCTCAG ACA

23

(2) INFORMATION FOR SEQ ID NO: 3

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3
CCGGTCCACT GTGCAAGAA 19
- (2) INFORMATION FOR SEQ ID NO: 4
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4
TCAGCTGCTC CAAATGCCGA AAGG 24
- (2) INFORMATION FOR SEQ ID NO: 5
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 20
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5
GAAGGTGAAG GTCGGAGTC 20
- (2) INFORMATION FOR SEQ ID NO: 6
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 20
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6
GAAGATGGTG ATGGGATTTC 20
- (2) INFORMATION FOR SEQ ID NO: 7
- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7
CAAGCTTCCC GTTCTCAGCC 20
- (2) INFORMATION FOR SEQ ID NO: 8
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8
TTCTCTGGAC TGAGGCTC 18
- (2) INFORMATION FOR SEQ ID NO: 9
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9
TCCCCTCCTC TCTGCTTT 18
- (2) INFORMATION FOR SEQ ID NO: 10
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10

AGACTCGGGC ATAGAGAT

18

(2) INFORMATION FOR SEQ ID NO: 11

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11

GGTTGAGACT CGGGCATA

18

(2) INFORMATION FOR SEQ ID NO:12

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12

TGAGGGTTGA GACTCGGG

18

(2) INFORMATION FOR SEQ ID NO: 13

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13

ACAGTTGAGG GTTGAGAC

18

(2) INFORMATION FOR SEQ ID NO: 14

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14
GGTGACAGTT GAGGGTTG 18

(2) INFORMATION FOR SEQ ID NO: 15
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15
GCAGTGTGGC AGCGGCAG 18

(2) INFORMATION FOR SEQ ID NO: 16
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16
AGGGCAGTGT GGCAGCGG 18

(2) INFORMATION FOR SEQ ID NO: 17
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17

CTCAGGGCAG TGTGGCAG

18

(2) INFORMATION FOR SEQ ID NO:18

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18

TTGGGCTCAG GGCAGTGT

18

(2) INFORMATION FOR SEQ ID NO: 19

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19

CATTTGGGCT CAGGGCAG

18

(2) INFORMATION FOR SEQ ID NO: 20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20

GTCAGGCACG GTGGAGAG

18

(2) INFORMATION FOR SEQ ID NO: 21

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21
GCAGGTCAGG CACGGTGG 18
- (2) INFORMATION FOR SEQ ID NO: 22
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22
GCAGCAGCAG GTCAGGCA 18
- (2) INFORMATION FOR SEQ ID NO: 23
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23
AGCGGCAGCA GCAGGTCA 18
- (2) INFORMATION FOR SEQ ID NO: 24
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24
CACCAGCGGC AGCAGCAG 18

(2) INFORMATION FOR SEQ ID NO: 25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25

CAGGAGCACC AGCGGCAG

18

(2) INFORMATION FOR SEQ ID NO: 26

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26

TATATTCCCA CCAACAGC

18

(2) INFORMATION FOR SEQ ID NO: 27

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27

TCTTCTCCCT GTCCCCTA

18

(2) INFORMATION FOR SEQ ID NO: 28

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28
ATTATTTTGA GGGTGGAT 18
- (2) INFORMATION FOR SEQ ID NO: 29
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29
GTTTCCTTTGT GGCACCTG 18
- (2) INFORMATION FOR SEQ ID NO: 30
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30
AGTAGGTTCC TTTGTGGC 18
- (2) INFORMATION FOR SEQ ID NO: 31
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31
GCCTGGACAG TCATTGTA 18

(2) INFORMATION FOR SEQ ID NO: 32

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32

CAGTCCGTAT CCTGCCCC

18

(2) INFORMATION FOR SEQ ID NO: 33

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33

AGCCGCTCTC AACTCCC

18

(2) INFORMATION FOR SEQ ID NO: 34

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34

TCTGAAGCGG TGAAGGAG

18

(2) INFORMATION FOR SEQ ID NO: 35

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35
GGTTTTCTGA AGCGGTGA 18
- (2) INFORMATION FOR SEQ ID NO:36
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36
AGGTGGTTTT CTGAAGCG 18
- (2) INFORMATION FOR SEQ ID NO: 37
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37
GTCTGAGGTG GTTTTCTG 18
- (2) INFORMATION FOR SEQ ID NO: 38
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38
AGGCAGTGTC TGAGGTGG 18

(2) INFORMATION FOR SEQ ID NO: 39

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39

AGCTGAGGCA GTGTCTGA

18

(2) INFORMATION FOR SEQ ID NO: 40

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40

CATTCCTTT CGGCATTT

18

(2) INFORMATION FOR SEQ ID NO: 41

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41

GACCCATTTC CTTTCGGC

18

(2) INFORMATION FOR SEQ ID NO: 42

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42
CACCTGACCC ATTCCTT 18
- (2) INFORMATION FOR SEQ ID NO: 43
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43
GGTACTGGTT CTCCTGC 18
- (2) INFORMATION FOR SEQ ID NO: 44
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44
TTTTCACTCC AATAATGC 18
- (2) INFORMATION FOR SEQ ID NO: 45
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45
CCATTGAGGC AGAGGCTG 18
- (2) INFORMATION FOR SEQ ID NO: 46

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46

ACGGTCCCAT TGAGGCAG

18

(2) INFORMATION FOR SEQ ID NO: 47

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47

ACGGTGTTCT GTTTCTCC

18

(2) INFORMATION FOR SEQ ID NO: 48

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48

CTACAGGAGA CACTCG

18

(2) INFORMATION FOR SEQ ID NO: 49

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49
CTTACAGTTA CTACAGGA 18

(2) INFORMATION FOR SEQ ID NO: 50

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50
GCTTTTCTTA CAGTTACT 18

(2) INFORMATION FOR SEQ ID NO: 51

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51
TCCAGGCTTT TCTTACAG 18

(2) INFORMATION FOR SEQ ID NO: 52

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52
TAACATTCTC AATCTGGG 18

(2) INFORMATION FOR SEQ ID NO: 53

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53
ACTGTGGTGC CTGAGTCC 18
- (2) INFORMATION FOR SEQ ID NO:54
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54
CAAAGACCAA AGAAAATG 18
- (2) INFORMATION FOR SEQ ID NO: 55
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55
AAAGGCAAAG ACCAAAGA 18
- (2) INFORMATION FOR SEQ ID NO: 56
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56

AGGAGGGATA AAAGGCAA

18

(2) INFORMATION FOR SEQ ID NO: 57

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57

CAATGAAGAG GAGGGATA

18

(2) INFORMATION FOR SEQ ID NO: 58

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58

TTAAACCAAT GAAGAGGA

18

(2) INFORMATION FOR SEQ ID NO: 59

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 59

CCGTTGGTAG CGATACAT

18

(2) INFORMATION FOR SEQ ID NO: 60

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 60
TCGATTTCCTCC ACAACAA 18
- (2) INFORMATION FOR SEQ ID NO: 61
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 61
CTTAGTAGTA GTTCCTTC 18
- (2) INFORMATION FOR SEQ ID NO: 62
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 62
GAAGCCTGGA GTGGGACT 18
- (2) INFORMATION FOR SEQ ID NO: 63
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 63

GGACTGAAGC CCAGGGTG

18

(2) INFORMATION FOR SEQ ID NO: 64

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 64

GTGGAACTGG GCACGGGA

18

(2) INFORMATION FOR SEQ ID NO: 65

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 65

TGAAGGTGGA ACTGGGCA

18

(2) INFORMATION FOR SEQ ID NO: 66

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 66

AGCTGGAGGT GAAGGTGG

18

(2) INFORMATION FOR SEQ ID NO: 67

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 67
CGCAAAGTTG GGACAGTC 18
- (2) INFORMATION FOR SEQ ID NO: 68
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 68
GTGCCACCTC TCTGCGGG 18
- (2) INFORMATION FOR SEQ ID NO: 69
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 69
CTGTCTCTCC ACTTCTGA 18
- (2) INFORMATION FOR SEQ ID NO: 70
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 70

AGGCTCTGTG GCTTGTGG

18

(2) INFORMATION FOR SEQ ID NO: 71

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 71

TCGTGGTCGC TCAGCCCT

18

(2) INFORMATION FOR SEQ ID NO:72

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 72

CCGCCTCCAG GTCGCCAG

18

(2) INFORMATION FOR SEQ ID NO: 73

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 73

GCAGCCCAGC AGGTCCAT

18

(2) INFORMATION FOR SEQ ID NO: 74

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 74
TCCTCCAGGC AGCCCAGC 18
- (2) INFORMATION FOR SEQ ID NO: 75
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 75
ATCTGAGAAG ACTGGGCG 18
- (2) INFORMATION FOR SEQ ID NO: 76
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 76
GCTCCTGCTT GCCCCTGC 18
- (2) INFORMATION FOR SEQ ID NO: 77
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 77
GTTAGCACCA AGTAGGCG 18

(2) INFORMATION FOR SEQ ID NO:78

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 78

CGCAAACCAC CCACTCAG

18

(2) INFORMATION FOR SEQ ID NO: 79

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 79

ATCCTCGCAA ACCACCCA

18

(2) INFORMATION FOR SEQ ID NO: 80

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 80

ATAGCGTCCC TCATCCTC

18

(2) INFORMATION FOR SEQ ID NO: 81

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 81
CTCAGGGACG AACCAGGG 18
- (2) INFORMATION FOR SEQ ID NO: 82
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 82
AAAGGCTCAG GGACGAAC 18
- (2) INFORMATION FOR SEQ ID NO: 83
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 83
ACAAAACAAA ACAAACA 18
- (2) INFORMATION FOR SEQ ID NO: 84
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 84
GCCAAGTTTC TATTAGTG 18
- (2) INFORMATION FOR SEQ ID NO: 85

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 85

GCAGAGGGCA CAGGAGTG

18

(2) INFORMATION FOR SEQ ID NO: 86

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 86

GTCCAGGCAG AGGGCACA

18

(2) INFORMATION FOR SEQ ID NO: 87

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 87

GCTTGTCCAG GCAGAGGG

18

(2) INFORMATION FOR SEQ ID NO: 88

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 88
TGCCTTAGGA CAGTTCAG 18

(2) INFORMATION FOR SEQ ID NO: 89

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 89
TCCGTGCTCG CCCCTGCC 18

(2) INFORMATION FOR SEQ ID NO: 90

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 90
TTGTTCCGTG CTCGCCCC 18

(2) INFORMATION FOR SEQ ID NO: 91

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 91
AGGCCCCATT GTCCGTG 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13763

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C12Q 1/68; C12N 15/85, 15/63; A61K 35/00

US CL :435/6, 320.1, 325, 366; 514/44; 536/23.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 320.1, 325, 366; 514/44; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, STN, MEDLINE, EMBASE, CAPLUS, BIOSIS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------------|---|--|
| X ----- Y | OJWANG et al. Modified Antisense Oligonucleotides Directed against Tumor Necrosis Factor Receptor Type I Inhibit Tumor Necrosis Factor alpha-Mediated Functions. Biochemistry. 1997, vol. 36, pages 6033-6045, see entire document. | 1, 2, 5-7, 9, 11, 12, 14, 15 ----- 3, 4, 8, 10, 13 |
| Y | AKHTAR et al. Interactions of antisense oligonucleotide analogs with phospholipid membranes. Nucleic Acids Research. 1991. vol. 19, no. 20, pages 5551-5559, see entire document. | 3, 4, 8, 10, 13 |
| Y | SANGHVI. Heterocyclic Base Modifications in Nucleic acids and Their Applications In Antisense Oligonucleotides. Antisense Research and Applications. 1993. pages 273-288, see entire chapter. | 3, 4, 8, 10, 13 |



Further documents are listed in the continuation of Box C.



See patent family annex.

| | |
|---|--|
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| *E* earlier document published on or after the international filing date | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *G* document member of the same patent family |
| *O* document referring to an oral disclosure, use, exhibition or other means | |
| *P* document published prior to the international filing date but later than the priority date claimed | |

Date of the actual completion of the international search

24 AUGUST 1999

Date of mailing of the international search report

21 OCT 1999

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13763

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y,P | US 5,789,573 A (BAKER et al) 04 August 1998, see entire patent. | 3, 4, 8, 10, 13 |
| X | SHEN et al. Inhibition of p75 Tumor Necrosis Factor Receptor by Antisense Oligonucleotides Increases Hypoxic Injury and beta-Amyloid Toxicity in Human Neuronal Cell Line. Journal of Biological Chemistry. February 7, 1997, vol. 272, no. 6, pages 3550-3553, see entire document. | 1, 2, 5, 6, 15 |